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Bronchiolo-Alveolar Carcinoma: An Analysis of Survival Predictors

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Macroscopic and microscopic features of tumours have been analysed in 37 bronchiolo-alveolar carcinomas. Lymphocytes, Langerhans cells, collagen (mature and/or myofibroblastic), were quantitatively or semiquantitatively evaluated. Histology, stage, type of fibrosis, nuclear profile features (area and shape factors), amount and type of mucin secreted, number of mitoses, Langerhans cells, myofibroblasts and LeuM1+ cells were not related to survival. Gross morphology of the tumour and, to a lesser extent, lymphoid infiltrates (in particular UCHL1+ and L26+ peritumoral lymphoid cells) were the only variables significantly related to survival. Estimated survival functions were computed according to Cox's model: well demarcated tumours behaved significantly better than poorly demarcated tumours and even more so than diffuse or multiple mass. Lymphoid infiltrates were significantly more represented in and around well demarcated tumours: however, their survival predicting value was less than that of the gross type.
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INTRODUCTION

SINCE THE description of Malassez [1], many microscopic and ultrastructural observations have been made regarding the nature of cells from which bronchiolo-alveolar carcinomas (BAC) originate [2–5]. A number of divergent terms have been used for their definition (to call them synonyms is absurd): diffuse epithelial hyperplasia, benign alveolar cell tumour of the lung or pulmonary adenomatosis, solitary or diffuse or multiple (multicentric) bronchiolar carcinoma and mucocellular (gelatinous) papillary adenocarcinoma. This implies controversy in histogenesis, differentiation, morphological and clinical presentation.

Extensive studies of large series of cases tend to keep two relatively distant entities separate within the group. The first consists of cases which prevalently exhibit Clara cell differentiation and in a minority of cases type II pneumocyte differentiation, with a predominantly alveolar growth pattern. This is associated with focal or dense sclerosis, often as a central area with entrapped tumour cells (so-called non-mucinous BAC). The other entity (rarer) is characterised by an exclusive bronchiolo-alveolar growth pattern, without fibrosis and/or inflammation, and by evident mucin production (apical cytoplasmic vacuoles containing mucin and frequent mucin flooding alveolar spaces, so-called mucinous BAC). It has been assessed that non-mucinous BACs are predominantly solitary lesions with a 5-year survival rate of 72%, whereas mucinous BAC are more frequently diffuse with a 5-year survival rate of 26% [6]. Non-mucinous BACs seem to elicit a B-lymphocytic immune response, mucinous, a T-lymphocytic response with Langerhans cells (LC) [7].

In this study, a number of BACs have been evaluated by employing morphometrical and immunohistochemical methods besides the conventional macroscopical and histological criteria.

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PATIENTS AND METHODS

The study group consisted of 37 cases deriving from an original group of 62 consecutive (38 males, 24 females) surgically treated lung tumours diagnosed as BAC between 1978 and 1989 at the Institute of Pathological Anatomy and Histopathology of the University of Siena. They represent about 10% of all lung tumours, and about 17% of adenocarcinomas observed in that period. 15 cases were lost to follow-up, 5 patients died due to postsurgical complications and 5 cases did not fulfill histological criteria of BAC at the revised diagnosis. 29 patients were male, 8 female; age ranged from 36 to 72 years (mean 57). Follow-up lasted from 2 to 61 months.

No chemotherapy and/or radiotherapy was adopted in the postsurgery period. Relapse of disease occurred in 24 patients: in all these cases chemotherapy was performed, however, all the patients died after a maximum of 21 months (mean: 6 months).

According to Axiotis and Jennings [7] the criteria for acceptance of a tumour as BAC were: (a) peripheral location, (b) absence of demonstrable bronchogenic origin, (c) well differentiated histology, (d) characteristic spreading pattern along pre-existing alveolar septa, and (e) absence of primary adenocarcinoma elsewhere.

All the cases accepted as BAC were histologically classified according to Manning *et al.* [6] and Clayton [8, 9] (Figs 1 and 2) and staged according to Mountain [10].

Gross features were recorded as follows: well-demarcated mass (group A), poorly demarcated mass (group B), diffuse or multiple mass (group C).

According to Clayton [8], aerogenous dissemination is a macroscopical feature. Tumours with multiple foci or very ill-defined borders, resembling pneumonia, are those presenting it. According to Manning *et al.* [6], aerogenous spread is a microscopical feature, meaning tumours utilising alveolar septa for supporting lattice work. As such, it may be encountered

both in a well-demarcated, solitary mass and in a poorly defined or multiple mass.

It must be emphasized that histologically assessed aerogenous spread is one of the criteria adopted by Watson [5], Axiotis and Jennings [7] and Grover *et al.* [11] for acceptance as BAC. Aerogenous spread defined as "tumour cell growing along the walls of the alveoli" is partially or diffusely present in all our BACs. For this reason, there is not a special section in our tables dedicated to aerogenous spread. According to Clayton's [8] macroscopical concept, all our cases grossly belonging to group A do not show aerogenous dissemination, while all cases belonging to group B and C present this spreading attitude.

Tumour tissue was fixed in 10% neutral formalin (pH 6.8–7.2) and paraffin embedded. Five micrometer sections were stained by the following methods: haematoxylin–eosin (H&E), mucicarmine, periodic acid Schiff (PAS) with and without diastase pretreatment, high iron diamine/alcian blue (HID/AB), Mallory, van Gieson and Gomori. The following antigens were investigated by means of immunohistochemistry (APAAP procedure): LeuM1 (Becton–Dickinson; 1:20), UCHL1 (pan-T) (Dakopatt's; 1:100), L26 (pan-B) (Dakopatt's; 1:100), CD1 (for Langerhans cell, LC [12]) (Dakopatt's; 1:100), smooth muscle actin (for myofibroblasts) (Sclavo, Siena; 1:100).

The degree of mucicarmine, PAS, HID/AB, Mallory, van Gieson and Gomori positivity was semiquantitatively scored from − to +++, as well as the entity of lymphocytic infiltrate

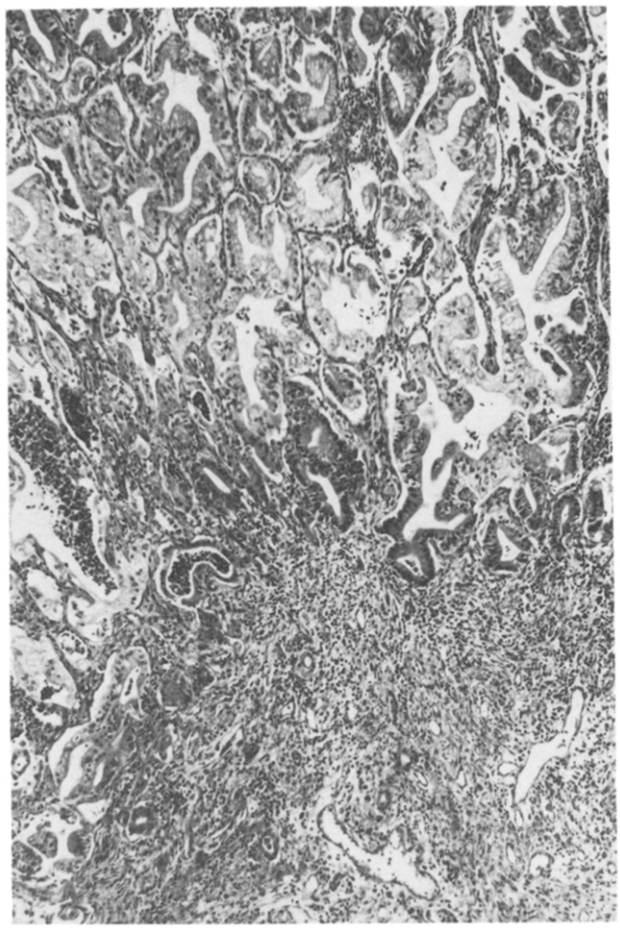

Fig. 1. Intermediate BAC: dense fibrosis with bordering non-mucinous neoplastic tissue with a prevalently alveolar growth (bottom); a mucinous pattern with a typical bronchiolo-alveolar growth (top).

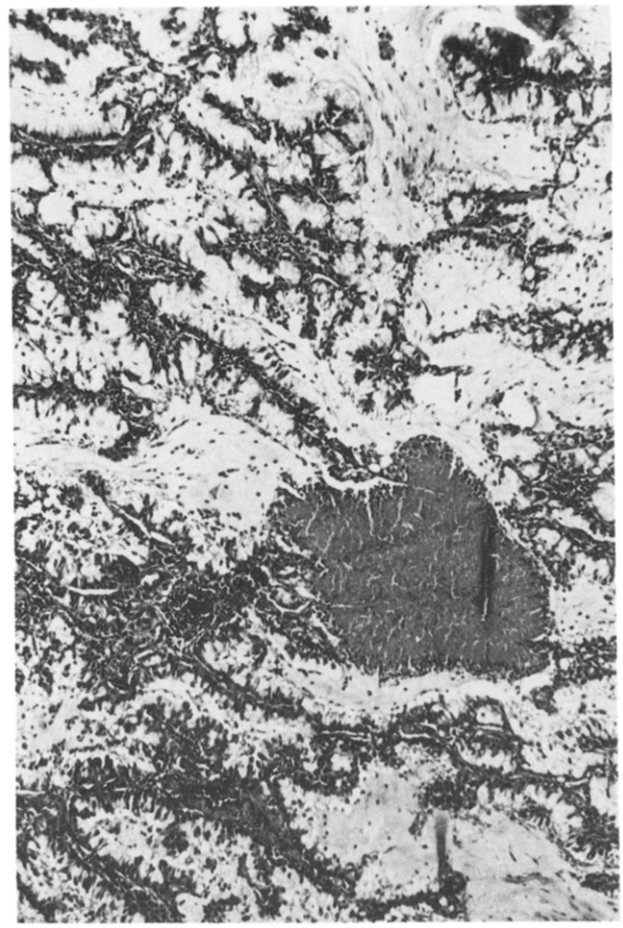

Fig. 2. Typical cytoarchitectural pattern of mucinous BAC.

(on H&E stained sections) and LeuM1 immunoreactivity. The population of UCHL1+ and L26+ cells was expressed as mean numbers in 10 peritumoral and intratumoral high power fields (HPF, objective × 100); the overall mean was also computed. To avoid selection of fields, cells were counted in 1 out of 5 consecutive HPFs. The population of CD1+ LCs was evaluated by counting positive cells in 10 randomly chosen HPFs. Myofibroblastic cells were expressed as the percentage of stromal cells positive to smooth muscle actin, not considering vessel smooth muscle cells [13]. Mitotic counts were registered as mean numbers of clearly defined mitotic figures in 10 randomly chosen HPFs totally occupied by neoplastic cells.

Morphometric analysis was performed on H&E stained sections (5 μm). A commercially available semiautomated interactive image analyser (IBAS KONTRON) was used, which consists of a magnetic tablet connected with a microcomputer and light-emitting diode (LED). The sections were observed by means of a microscope equipped with a camera lucida (final enlargement 1800×): such magnification was chosen to obtain an acceptable reproducibility [14]. Using the LED projected into the microscopic field under study, the boundaries of 100 randomly selected neoplastic nuclei were accurately outlined. Fifty nuclei were sufficient to have intra-observer and inter-observer coefficients of variation of measurements within the limits of 5%. However, to be on the safe side, 100 nuclei per case were measured, excluding necrotic, overlapping or not clearly defined nuclei. Measurements were recorded, and in each case the mean and SD of the following nuclear profile

parameters were calculated: area, ellipsoidity (form E11), regularity (form Ar) and roundness (form Pe).

Form E11 is an elongation factor calculated by the following formula: (minor diameter/major diameter) × 1000. Its value is 1000 for a circle and less than 1000 for elliptical structures. Form Ar is calculated by:

$$\frac{\text{area}}{\pi / 4 \times \text{major diameter} \times \text{minor diameter}} \times 1000$$

and is = 1000 for a circle and ellipse, less than 1000 for irregular structures.

Form Pe is calculated by:

$$(4\,\pi \times \text{area} / \text{perimeter}^2) \times 1000.$$

Its value is 1000 for a circle and less than 1000 for elliptical and irregular structures.

Reproducibility

A complete agreement was reached between the observers (V.S., R.S.) on the semiquantitative data, by discussing and rediscussing findings under the microscope. The reproducibility of results for all the quantitative parameters was assessed independently by the same observers in 8 cases by repeating the measurements seven times. The intra-observer and inter-observer coefficient variation values were maximally 5% for nuclear parameters, 8% for the other parameters.

Statistics

Statistical analysis was performed to quantify the relationship between patient survival and corresponding set of explanatory variables (covariate vector x). Analysis was based upon Cox's [15] proportional hazards regression model, so modelling the death rates as log-linear functions of the covariate vector [16]. To this end, a stepwise selection algorithm was used in an explanatory manner to identify a subset of independent variables significantly related to survival, because, in the case considered, a large number of independent variables were candidates for inclusion in the model. In this way at each step, one variable was entered or removed from the model on the basis of a computed significance probability. In particular, the selection of each variable for entry or removal was based on the maximum partial likelihood ratio test [17].

The list of measured variables which were considered as candidates for inclusion in the model is shown in Table 1.

At the end of the stepwise selection, plots of survival functions were obtained for different values of most significant variables. As a result of this analysis, four variables were selected (gross tumour appearance, amount of lymphoid infiltrates—semiquantitatively evaluated inside and at the periphery of the tumour—total number of UCHL1+ lymphoid cells, peritumoral L26+ lymphoid cells), which were most significantly related to survival function.

Linear correlation was tested between semiquantitatively scored lymphocytic infiltrate and quantitatively assessed UCHL1+ and L26+ cells.

All statistical computations were performed by BMDP [17] running a DEC microVAX II computer. $P < 0.05$ was adopted as the level of significance.

RESULTS

Clinical, gross, histological, semiquantitative and quantitative data of lymphoid infiltrates are reported in Tables 2 and 3.

Table 1. Variables considered in the analysis

1	Gross type
2	Stage
3	Histotype
	Type of mucin
4	PAS with diastase pretreatment
5	PAS without diastase pretreatment
6	Mucicarmine
7	HID/AB
	Type of fibrosis
8	Mallory
9	van Gieson
10	Gomori (recent vs mature collagen)
	Quantitative and semiquantitative data
11	Nuclear area
12	Standard deviation of nuclear area
13	Form E11
14	Form Pe
15	Form Ar
16	Mitotic count
17	Leu-M1+ cells (semiquantitative)
18	Langerhans cells
19	Lymphoid cell infiltrate (semiquantitative)
20	UCHL1+ peritumoral lymphoid cells
21	UCHL1+ intratumoral lymphoid cells
22	UCHL1+ cells, overall mean
23	L26+ peritumoral lymphoid cells
24	L26+ intratumoral lymphoid cells
25	L26+ cells, overall mean
26	Lymphoid cell infiltrate, total
27	Myofibroblasts

9 of the 37 cases presented morphological features intermediate between mucinous and non-mucinous cancers: mucinous and non-mucinous (PAS-, mucicarmine-, HID/AB-negative) areas, non-mucinous macroscopically diffuse patterns, and others. Pure mucinous cases were only 3, non-mucinous cases were 25. For this reason, cases to be correlated with survival were considered as one group with regard to histological, histochemical, immunohistochemical and quantitative findings.

By means of a stepwise selection algorithm, a subset of four independent variables significantly related to survival and giving the best estimation of survival function (according to Cox's model) [15] was selected. The summary of stepwise analysis is shown in Table 4. No variable was removed by the analysis. The parameter regression vector estimated by the algorithm is shown in Table 5 together with its standard error.

The macroscopic appearance of the tumour is the most significantly survival-related parameter: group A cases (well-demarcated mass, $n = 18$) have the best estimated survival, group C (diffuse or multiple mass, $n = 7$) the worst, group B (poorly demarcated mass, $n = 12$) have intermediate estimated survival. Lymphoid infiltrates (semiquantitatively scored), UCHL1+ and L26+ peritumoral lymphoid cells have minor although significant influence on estimated survival function. In fact, by evaluating only gross tumour features in individual cases, and by considering the other parameters as constant (equal to their mean values), it is possible to obtain estimated survival functions which are highly significantly different among groups A, B, C. Significant differences are shown in each gross group by using the other three variables: lymphoid infiltrates (semiquantitatively evaluated), UCHL1+ and L26+ peritumoral lymphoid cells.

The estimated survival function resulting from computing values of these lymphoid cell-related variables in single cases is significantly better in cases with well-demarcated tumours (group A) than in cases with poorly defined masses (group B), and better in these latter cases than in patients with diffuse or multiple mass (group C).

Stage, histotype, mitotic count, number of LC, myofibroblasts, LeuM1+ cells and all the semiquantitative evaluated parameters (with the exception of lymphocytic infiltrates) together with nuclear profile morphometric features do not significantly add to the estimated survival functions.

As expected, a significant linear correlation exists between semiquantitatively scored lymphocytic infiltrate and quantitatively evaluated UCHL1+ and L26+ cells: UCHL1+ peritumoral cells: $r = 0.789$, $P < 0.001$; intratumoral: $r = 0.298$, $P < 0.05$; overall mean: $r = 0.685$, $P < 0.001$; L26+ peritumoral cells: $r = 0.659$, $P < 0.001$; intratumoral: $r = 0.428$, $P < 0.01$; overall mean: $r = 0.596$, $P < 0.001$.

Table 2. Clinical and histological data in individual cases

No.	Age	Sex	Survival (months)	Alive/ dead	Gross type	Stage	Histotype
1	36	M	2	D	DMM	IIIa	Intermediate
2	60	M	2	D	PDM	I	Non-mucinous
3	65	M	3	D	DMM	IIIa	Non-mucinous
4	53	M	4	D	PDM	II	Intermediate
5	64	M	5	D	DMM	IIIa	Non-mucinous
6	49	M	5	D	WDM	I	Non-mucinous
7	60	M	5	D	DMM	IIIa	Mucinous
8	40	M	7	D	DMM	IIIa	Non-mucinous
9	65	F	7	A	DMM	IIIa	I
10	53	M	9	D	WDM	I	Intermediate
11	51	M	10	D	PDM	I	Non-mucinous
12	49	F	12	A	WDM	I	Non-mucinous
13	57	F	16	A	WDM	I	Intermediate
14	44	M	16	D	PDM	I	Non-mucinous
15	70	M	17	D	DMM	IIIa	Intermediate
16	54	M	18	D	PDM	I	Non-mucinous
17	61	M	20	A	WDM	I	Non-mucinous
18	55	M	24	A	WDM	I	Non-mucinous
19	58	F	24	D	WDM	I	Intermediate
20	57	F	26	D	WDM	I	Non-mucinous
21	67	M	29	D	PDM	II	Non-mucinous
22	62	M	30	A	WDM	I	Non-mucinous
23	57	M	30	A	WDM	I	Intermediate
24	65	M	33	A	PDM	II	Non-mucinous
25	57	M	35	D	PDM	IIIa	Non-mucinous
26	60	M	36	A	WDM	I	Mucinous
27	69	M	36	A	WDM	I	Non-mucinous
28	64	M	37	D	PDM	IIIa	Non-mucinous
29	46	F	38	D	WDM	II	Non-mucinous
30	67	M	39	A	WDM	I	Non-mucinous
31	62	M	40	D	PDM	I	Non-mucinous
32	54	M	41	D	WDM	I	Non-mucinous
33	45	F	41	A	WDM	I	Non-mucinous
34	72	M	43	A	WDM	I	Intermediate
35	61	M	43	D	PDM	II	Non-mucinous
36	45	F	57	D	PDM	IIIa	Intermediate
37	67	M	61	D	WDM	I	Mucinous

M = male, F = female.
WDM = well demarcated mass, PDM = poorly demarcated mass, DMM = diffuse or multiple mass.

Table 3. Semiquantitative and quantitative data of lymphoid infiltrates in individual cases

		UCHL1			L-26			
No.	Lymphoid cell infiltrate*	†	‡	§	†	‡	§	‖
1	+	11	14	13	25	8	16	29
2	−	2	0	1	8	3	5	7
3	−	10	10	10	4	0	2	12
4	+	9	39	24	28	16	22	46
5	−	2	4	3	4	0	2	5
6	+	16	17	15	2	0	1	16
7	−	1	1	1	1	1	1	2
8	−	2	25	3	0	0	0	3
9	+++	26	4	15	38	30	34	49
10	+	15	25	20	6	0	3	23
11	+	32	44	38	4	0	2	40
12	++	16	6	12	8	0	4	16
13	+++	56	36	46	50	30	40	86
14	+	20	8	14	10	10	10	24
15	+	10	1	5	4	0	2	7
16	+	14	10	12	10	0	5	17
17	+	14	2	8	0	0	0	8
18	++	58	16	37	4	0	2	39
19	+	37	29	33	8	0	4	37
20	−	1	1	1	0	0	0	1
21	+	16	14	15	4	2	3	18
22	+++	35	30	33	40	20	30	63
23	+	22	15	18	4	0	2	20
24	+++	33	31	32	16	4	10	42
25	+++	29	10	20	20	4	12	32
26	−	5	1	3	0	0	0	3
27	+	14	0	7	0	0	0	7
28	+	14	6	10	4	0	2	12
29	++	22	18	20	8	2	5	25
30	++	20	0	10	8	0	4	14
31	++	37	26	32	10	2	6	38
32	+++	38	28	33	30	0	15	48
33	+++	33	7	20	26	4	15	35
34	++	24	7	16	16	0	8	24
35	++	25	8	17	4	0	2	19
36	+++	51	14	33	4	0	2	35
37	+	8	8	8	2	0	1	9

* Semiquantitatively evaluated.
† Peritumoral.
‡ Intratumoral.
§ Overall mean.
‖ Quantitatively evaluated lymphoid cell infiltrate, total.

DISCUSSION

Gross patterns and histological, histochemical, immunohistochemical and some quantitatively evaluated nuclear profile features of neoplastic cells were analysed in 37 BACs and data were related to survival.

9 of the 37 cases had to be classified as intermediate BAC, due to the presence of conflicting features in the same tumour. Only 3 cases were macroscopically and microscopically strictly mucinous cancers.

Cases were distributed quite haphazardly in accordance with survival, either considering two groups (mucinous, non-mucinous) or three groups (i.e. including intermediate cases). For this reason, it was decided to evaluate cases individually and not as histologically subtyped groups. Stage, histotype, type of growth, quantity and type of mucins secreted, fibrosis,

Table 4. Summary of stepwise results

Step No.	Variable entered	Degrees of freedom	Log likelihood	Improvement χ^2	*P* value	Global χ^2	*P* value
1	Gross features	1	−58.134	15.469	< 0.0001	16.956	< 0.0001
2	Lymphoid cell infiltrate	2	−52.640	10.989	0.001	28.746	< 0.0001
3	L26+ peritumoral lymphoid cells	3	−48.279	8.722	0.003	38.366	< 0.0001
4	UCHL1+ cells, overall mean	4	−45.293	5.973	0.015	41.017	< 0.0001

Table 5. Regression parameter vector of the variables significantly related to survival

Variable	Regression parameter	Standard error
Gross features	1.7453	0.4513
Lymphoid cell infiltrate	−2.4886	0.6023
L26+ peritumoral lymphoid cells	0.1130	0.0369
UCHL1+ cells, overall mean	0.0741	0.0293

LeuM1 immunoreactivity, nuclear profile features (area and shape factors), number of mitoses, LC, myofibroblasts did not influence the estimated survival function according to Cox's [15] proportional hazards regression model. Based upon a stepwise selection algorithm, four variables were significantly related to survival. Gross characteristics resulted to be of primary importance, well-demarcated tumours showing better behaviour than poorly demarcated tumours, and even more so than diffuse or multiple mass (Fig. 3). The other parameters of major significance were the amount of lymphoid infiltrates (semiquantitatively evaluated), the number of UCHL1+ lymphocytes and the number of peritumoral L26+ lymphocytes. In a group of cases harbouring tumours with the same gross features, the entity of lymphoid infiltrates (evaluated by means of the three above mentioned parameters) was strictly related to survival. However, the discrimination of gross features alone, i.e. when the lymphoid cell-related parameters were kept as constant, was significantly higher. It seems important to have demonstrated a good linear correlation between semiquantitatively scored lymphoid infiltrates and quantitatively assessed UCHL1+ and L26+ lymphoid cells. This brings us to the conclusion that evaluation of the number of lymphoid cells in and around the tumour can be routinely performed even without using measurement equipment.

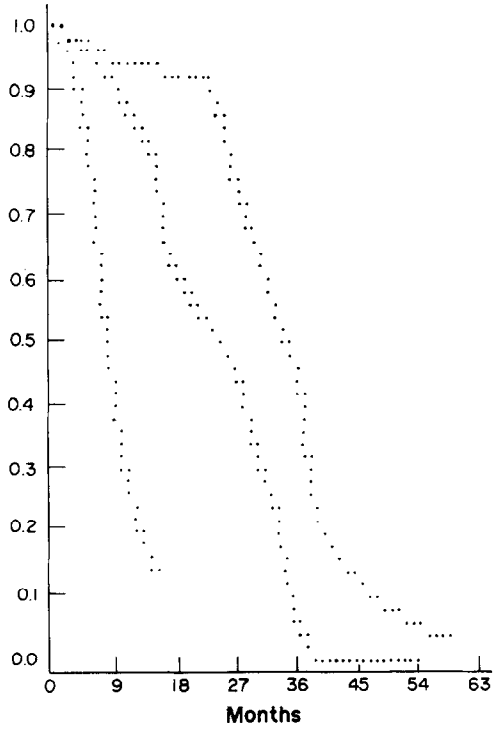

Fig. 3. Estimated survival functions among group A (well-demarcated mass), B (poorly demarcated mass), C (diffuse or multiple mass).

Our results are in contrast with previous reports [6, 8] and may be considered surprising, at least to a certain extent. Regarding the lack of a stage-survival relationship, it must be stressed that we are dealing with a relatively small number of cases and this may determine *per se* no significant differences among stages. It has to be added that our stage III cases are such in most instances because of parietal pleural involvement, only in few cases is it due to distant lymph node invasion. In our series, up to 27 out of 37 cases are node negative. As a further consideration, one might hypothesise, according to Mountain *et al.* [18] and Clayton [9], that uniform staging of pulmonary cancer are not adequate for all its types. In some of our stage I cases a sudden metastatic spread after a few months from the diagnosis (time at which metastases were not detectable) determined an early death of patients. Perhaps more important, our results question the assumption of subtypes of BACs as entities, in agreement with some other authors (for review, see Grover *et al.* [11]).

Our findings are in line, at least in part, with those of Lee *et al.* [19], who studied non-small cell lung cancers by means of both morphometrical and conventional morphohistological criteria. In that study the entity of lymphoid cell infiltrates (peritumoral and, to a minor grade, intratumoral) is the only factor with prognostic significance, while traditional morphologic assessments (stage, histology, fibrosis, mucin secretion) as well as morphometric nuclear profile features have limited, if any, usefulness in predicting the biological behaviour of the tumour. It means that the tumour–host interaction is an important prognostic discriminator, as shown in many other malignancies (for review, see Lee *et al.* [19]), and that the peritumoral area is often the site where the immune response is maximal [20–23].

Gross morphology of the tumour is the most important survival-related parameter in our study, however, it is immediately followed by lymphoid cell infiltrate. Well-demarcated

tumours present higher numbers of peritumoral and intratumoral lymphoid cells, poorly demarcated tumours and, more so, diffuse or multiple tumours show lower numbers of lymphoid cells. Maybe the expanding or infiltrative tumour growth is related to a more or less pronounced immunological response.

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A Pilot Study of Photodynamic Therapy in Patients with Inoperable Non-small Cell Lung Cancer

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26 patients with inoperable non-small cell lung cancer (NSCLC) were treated with photodynamic therapy (PDT) with intravenous Photofrin II 2 mg/kg. 10 out of 11 stage I patients achieved a complete response. The remaining patient, and 11 out of 15 stage III patients had a partial response. No response was seen in 4 patients, 2 of whom had inadequate illumination. Thus, the objective response rate was 85% (22/26). Although lung function did not improve, dyspnoea was ameliorated in 7 (58%) of the partial responders. 4 stage III patients had tumour progression and died of pulmonary haemorrhages 1.5–6 months after PDT. All had received external irradiation, Nd-YAG laser and/or brachytherapy before PDT. 4 patients had grade I–II skin photosensitivity. Although of value in stage I NSCLC, the clinical benefit of PDT in stage III disease was small.
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INTRODUCTION

PHOTODYNAMIC THERAPY (PDT) is based on the illumination of malignant tissue containing compounds (photosensitisers) activated by light. Illumination with light of an appropriate wavelength initiates a photochemical reaction and the formation of various radicals in the vicinity of the activated sensitiser molecule. This process leads to the disruption of biomolecular structures, causing vascular thrombosis and tissue necrosis [1–3]. PDT offers the possibility of selective tumour damage due to the local application of laser light and the relatively higher retention of photosensitisers in tumour stroma, in comparison to the surrounding normal lung tissue [3]. The experiences with PDT in lung cancer have suggested that palliation of locally advanced tumours is feasible [4–6], and that cure may be